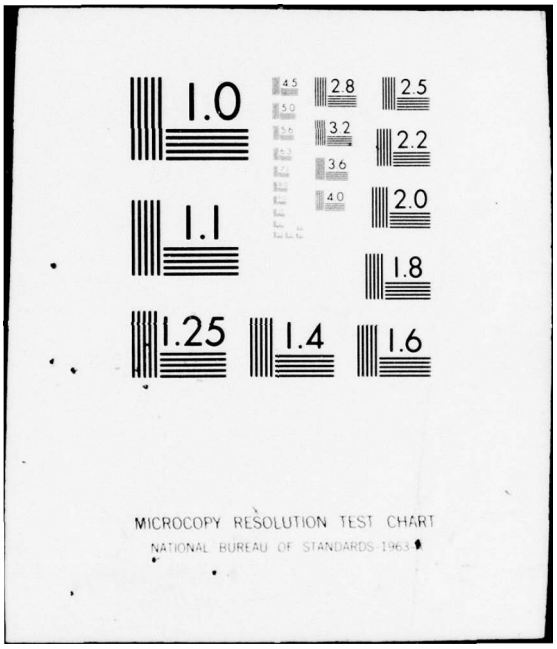
AD-A043 445 ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC F/G 6/1
DIFFERENTIAL PROTECTION OF MAMMALIAN CELLS FROM PSEUDOMONAS AND--ETC(U)
1977 J L MIDDLEBROOK, R B DORLAND

UNCLASSIFIED NL

1 OF 1
AD A043445

END
DATE FILMED
9-77
DDC


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

Differential Protection of Mammalian Cells from Pseudomonas and Diphtheria Exotoxins by Exogenous Nucleotides

JOHN L. MIDDLEBROOK and REBECCA B. DORLAND

Pathology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21701

Running Title: NUCLECTIDE EFFECTS ON EXOTOXIN CYTOTOXICITY

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle)
Differential Protection of Mammalian Cells from Pseudomonas and Diphtheria Exotoxins by Exogenous Nucleotides,

5. TYPE OF REPORT & PERIOD COVERED
Interim

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)
John L. Middlebrook and Rebecca B. Dorland

8. CONTRACT OR GRANT NUMBER(s)

9. PERFORMING ORGANIZATION NAME AND ADDRESS
U.S. Army Medical Research Institute of Infectious Diseases; Ft Detrick
Frederick, MD 21701
SGRD-UIP-A

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
61102
3M161102 BS03-00-019

11. CONTROLLING OFFICE NAME AND ADDRESS
U.S. Army Medical Research and Development Command, Office of The surgeon General
Department of the Army, Washington, DC 20314

12. REPORT DATE
11 1977

13. NUMBER OF PAGES
25 pages and 7 graphs
12 33 p.

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)
16 3M161102BS03
12 00

15. SECURITY CLASS. (of this report)
UNCLASSIFIED

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Reprints bearing assigned AD number will be forwarded upon receipt. To be submitted for publication in Infection and Immunity.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Pseudomonas toxin, diphtheria toxin, cytotoxicity protection, nucleotides.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Exogenous nucleotides were found to protect mammalian cells from Pseudomonas (PE) and diphtheria (DE) exotoxins. The protection was dose-dependent and required the simultaneous presence of nucleotide during toxin challenge. Protection from DE was proportional to the number of phosphates in the nucleotides, exhibited base specificity, and required an intact nucleotide for full expression. Protection from PE seemed to require only phosphate oligomers. Neither phosphorylation nor interference with the (intracellular) enzymatic activities of the toxins appeared to mediate the protection. It was concluded that exogenous nucleotides block the attachment or internalization stages of intoxication.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

AD A05039

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

ABSTRACT

Exogenous nucleotides were found to protect mammalian cells from Pseudomonas (PE) and diphtheria (DE) exotoxins. The protection was dose-dependent and required the simultaneous presence of nucleotide during toxin challenge. Protection from DE was proportional to the number of phosphates in the nucleotides, exhibited base specificity, and required an intact nucleotide for full expression. Protection from PE seemed to require only phosphate oligomers. Neither phosphorylation nor interference with the (intracellular) enzymatic activities of the toxins appeared to mediate the protection. It was concluded that exogenous nucleotides block the attachment or internalization stages of intoxication.

Corynebacterium diphtheriae, the causative organism of diphtheria, produces a single exotoxin (DE) which is believed to be responsible for the severe symptoms of the disease. DE is lethal for several animal species and cytotoxic for a variety of cultured mammalian cells (5, 9, 21). Cell culture studies indicate that the primary event leading to cytotoxicity is a toxin-induced inhibition of protein synthesis. Inhibition appears to result from toxin-catalyzed inactivation of an intracellular enzyme necessary for protein synthesis, elongation factor 2 (EF-2) (5,9,21).

Pseudomonas aeruginosa is a ubiquitous, normally harmless bacterium which can produce life-threatening infections in patients with impaired immune function (15). Unlike C. diphtheriae, P. aeruginosa produces several cytopathogenic extracellular products (15). However, there is increasing evidence that one of these, exotoxin A (PE), plays a particularly important role in the infection pathogenesis. For example, PE is produced by most clinical isolates (2, 24), cytotoxic for many mammalian cell lines (16, 22) and lethal for several animal species (14, 15, 23). The report that PE inhibits protein synthesis in mammalian cells (22), followed by a demonstration that PE has an enzymatic ADP-ribosylating activity similar to that of DE (11) raised the possibility that these two toxins have similar mechanisms of cellular intoxication. More recent evidence indicates that such is not the case and that, despite similar enzymatic activities, PE and DE somehow differ in their overall mechanisms of intoxication (16, 18).

We report here that exogenous nucleotides protect cells from the actions of both PE and DE. Although the characteristics of protection from each toxin are different, our results are

consistent with the view that the attachment or internalization stages of PE and DE intoxication are perturbed by nucleotides. This observation may provide a future probe into the mechanism by which both toxins enter the cell.

MATERIALS AND METHODS

Cells and cell culture. Seed stock for all cell lines was obtained from the American Type Culture Collection (ATCC), Rockville, Md. Each line was maintained in 75-cm^2 T-flasks (Costar #3075) with the medium and serum supplement recommended by ATCC.

Media and sera. All media, vitamins, antibiotics and amino acids were obtained from Grand Island Biological Company, Grand Island, N.Y. Fetal calf serum was purchased from Reheis Chemical Company, Phoenix, Ariz. The serum was heat-inactivated for 30 min at 56°C before use in cell culture.

Toxins. P. aeruginosa, strain PA103, was obtained from P. V. Liu. P. aeruginosa exotoxin A was produced and purified by Stephen Leppla of this Institute (14). The final product behaves as a single polypeptide of 66,000 daltons, contains no measurable carbohydrate and is more than 95% pure as judged by sodium dodecyl sulfate, polyacrylamide gel electrophoresis. Heating the purified toxin at 70°C for 1 h completely destroys its cytotoxic activity. The median lethal dose (LD_{50}) of PE purified in this manner was 0.1 μg/20-g CD-1 mouse. PE was "activated" for ribosylation experiments by a previously described method (14). DE was obtained from Connaught Laboratories, Toronto, and purified by chromatography over DE-52, G-100 Sephadex and hydroxylapatite. The final product was indistinguishable in cell culture experiments

from purified DE (23 MLD/μg) supplied by Dr. A. M. Pappenheimer, Jr., Harvard University. DE fragment A was prepared by a previously published method (8). Toxin concentrations were determined using extinction coefficients ($E_1^{1\%}$cm) at 280 nm of 10.5 and 11.9 for PE and DE, respectively.

Chemicals. Chemicals, their abbreviations and sources are listed in Table 1. Concentrations were determined spectrophotometrically, using either published extinction coefficients (29) or those furnished by the supplier; purity was assessed by thin layer chromatography (25). [^{14}C]Nicotinamide adenine dinucleotide was obtained from Amersham/Searle, Arlington Heights, Ill. [^{14}C]ATP, [^{14}C]ADP and [^{3}H]AMP were purchased from New England Nuclear, Boston, Mass.

Cytotoxicity assay. Details of our cytotoxicity assay have been recently described (17); a slight variation of the method was used for this work. Toxin and the chemical agent under investigation were added simultaneously to cells in multi-well tissue culture plates (triplicate samples) and incubation was carried out at 37°C for 3 h. All nucleotide solutions were prepared in a 0.1 M N'-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES) buffer and adjusted to pH 7.2 ± 0.2 prior to addition to cells. The incubation was terminated by washing each monolayer three times with serum-free medium and adding complete medium to continue cell culture. After 48 h of further incubation the monolayers were washed with Hanks' balanced salt solution and the remaining adherent cells were dissolved in 0.1 M NaOH for protein assay. Data are plotted as percent of control incubations with the chemical agent alone; standard errors were usually 1-4% (16).

Ribosylation assay. The effects of chemicals on ADP-ribosyl transferase activity of "activated" PE and DE fragment A were carried out as previously described (16).

RESULTS

Protection from PE and DE by adenine nucleotides. Exogenous adenine nucleotides protected cells from both PE and DE although the pattern of specificity was different for each toxin. Protection was dose-dependent and related to the length of the phosphate chain on the nucleoside. Adenine nucleotide protection of HeLa cells from DE is demonstrated in Figure 1A. A4P was the most potent protective agent of the series, about 10-fold more potent than ATP. ATP was three to four times more potent than ADP, while AMP exhibited a low, but measurable level of protection. Neither the nucleoside, adenosine, at concentrations up to 5 mM, nor the free base, adenine, at concentrations up to 1 mM, exhibited protective potential.

Although HeLa cells also respond to PE (18), they are about 100-fold less sensitive to this toxin than to DE. We were unable to detect adenine nucleotide-mediated protection of HeLa cells from PE, even using relatively low doses of PE coupled with high (5 mM) concentrations of the protective agents (data not shown). However, we did observe protection in a much more PE-sensitive cell line, L-929 (18). A4P, ATP, ADP and AMP all protected L-929 cells from PE (Fig. 1B). Although protective potency seemed roughly proportional to phosphate chain length, the relationship was considerably less well defined than that seen with DE (Fig. 1A). Adenosine or adenine were without effect on PE-induced cytotoxicity.

Phosphate oligomers provided some measure of protection from PE and DE. Figure 2A shows the effect of pyrophosphate and tripolyphosphate on DE-challenged HeLa cells. Although both compounds were active, tripolyphosphate was only 1/10 and pyrophosphate 1/50 as potent as ATP. Neither phosphate compound measurably affected PE-induced cytotoxicity in HeLa cells. Protection from PE was apparent, however, in the more toxin-sensitive L-929 cell line. Pyrophosphate and tripolyphosphate both protected L-929 cells from PE, although in this case their potencies were not significantly less than that of ATP (Fig. 2B), a markedly different pattern of specificity from that obtained with DE (Fig. 2A).

Specificity of the nucleotide base. Nucleotide-mediated protection from PE and DE was not limited to adenine nucleotides. Figure 3 shows that all the common purine and pyrimidine ribonucleotides protected cells from both toxins. In DE-treated HeLa cells (Fig. 3A), TTP was consistently the most potent protective nucleotide and CTP the least potent, not demonstrably different from tripolyphosphate (compare Figs. 3A and 2A). The potencies of GTP, ITP and UTP varied slightly from experiment to experiment but were not reproducibly different from that of ATP. Nucleotide-mediated protection of L-929 cells from PE did not show this pattern of specificity: all the nucleotide triphosphates were equipotent (Fig. 3B) and not significantly different from tripolyphosphate.

Protection from DE by nucleotide components. The presence of nucleotide components is not sufficient to achieve full DE protection as was demonstrated by the experiment depicted in Figure 4. Here, TTP provided a high level of protection from a concentration of DE leading

to substantial cell death. Tripolyphosphate and thymidine separately provided no protection, and together, their protective potential was not increased. Thus, it appears that covalent attachment of tripolyphosphate to the nucleoside is necessary for full expression of DE-protective activity.

Structure-activity relationships of the DE-protective compounds. Structure-activity relationships of the DE-protective compounds were examined and experimental results are listed in Table 2. For the sake of simplicity, the data are presented on a +++, ++, +, 0 scale using ATP as a reference standard: +++ is equal to or greater than ATP protective potency, ++ is less potent than ATP but still substantially active, + is barely measurable, and 0 ineffective.

Part A lists nucleotide compounds with different sugar moieties. Each of the 2-deoxyribonucleotides exhibited DE-protective potency indistinguishable from that of its corresponding ribonucleotide. Similarly, substitution of arabinose for ribose in ATP and CTP did not measurably change their protective potential.

Part B demonstrates that halogenated or hydroxylated analogs of the purine or pyrimidine bases generally are not altered in protective potential relative to the parent compound. Single bromine replacements in ATP, dUTP or dCTP did not increase or decrease protective potency; similarly, trifluorination of TTP did not change protective activity. Xanthine triphosphate, a 2,6-dihydroxy congener of ATP or GTP, was slightly reduced in potency compared to either parent compound.

Part C shows the protective activities of dinucleotides, polynucleotides and cylic nucleotides. The dinucleotides ApA, ApC

and CpA were not measurably different from AMP, offering only low level protection, as did Poly A and Poly C. Neither cAMP, cGMP nor their dibutyryl derivatives gave measurable protection, nor did the phosphodiesterase inhibitor, theophylline, either alone or in combination with the cyclic compounds (data not shown).

Part D lists compounds not conveniently classified in one of the above groups. ADPR and PRPP conferred a low level of protection from DE. Acetyl CoA also provided a low level of protection from DE, although CoA had no measurable effect. Neither NAD, its fluorescent derivative, εNAD, nor the polyanion, heparin, had measurable protective potency. IHP and IHS were both very effective protectors, IHS as potent as A4P, and IHP between A4P and ATP in potency. The monophosphate of inositol, IP, was barely active and inositol itself exhibited no protective potential.

Kinetics of protection. In the previously described experiments, protection was obtained by the simultaneous addition of nucleotide and toxin to the test cell populations. In the following studies, the effect of nucleotide preincubation with either cells or toxin was investigated (Fig. 5). Preincubation of cells with ATP for as long as 2.5 h prior to toxin challenge (open circles, 2.5 to 0 h) did not change the protective level relative to that obtained by the simultaneous addition of ATP and toxin (open circle, 0 h). If toxin were incubated with ATP at 37°C prior to addition to cells (open triangles), an apparent small increase in protective potency was noted. This increase is probably not ATP-mediated, however, since the control incubation of toxin alone at 37°C showed a similar increase (closed triangles), indicating a temperature-induced loss

of cytotoxic activity. ATP addition to cells after various periods of toxin challenge resulted in rapidly decreasing protective potential with increasing challenge time. In as little as 1/2 hour, a marked reduction in apparent protective potential was observed (closed circles) and by 3 h, no measurable protection could be obtained. Plotted in a semilog fashion, these data exhibit apparent first-order kinetics, essentially like those obtained when the cell monolayers are simply washed after various challenge periods (18).

Nonhydrolyzable ATP analogs. The correlation of nucleotide phosphate chain length to protective potency suggested that DE protection may be mediated by phosphate transfer. To test this possibility, we investigated the protective effects of several nonhydrolyzable analogs of ATP. Results are shown in Figure 6. In the presence of ATP, cells are effectively protected from a concentration of DE that ordinarily kills about 70% of the cell population. ATP analogs with methylene linkages between the α-β (AMP-MPP) or β-γ (AMP-PMP) phosphates were similar in potency and slightly less protective than ATP. A third analog (AMP-PNP) with an imide linkage between the β-γ phosphates was equipotent to ATP. These results indicate that phosphate transfer is not responsible for the DE-protective effect of ATP.

Binding of nucleotides to DE. In an attempt to elucidate the mechanism by which nucleotides protect cells from DE, we investigated the binding of various compounds of the adenosine phosphate series to DE. Initially, we measured the binding of radiolabeled ATP, ADP and AMP to DE by equilibrium dialysis. Low level binding was observed, but it was not saturable and was not blocked by large

excesses of the corresponding unlabeled compounds. Moreover, in contrast to their varied protective potencies (see Fig. 1), the binding levels of all three nucleotides were essentially the same.

Effects of nucleotides on ADP-transferase activity. The effects of adenine, adenine nucleotides, and adenosine on DE- and DE fragment A-catalyzed ribosylation have been previously described (10, 12). We tested the effects of these compounds on PE- and "activated" PE-catalyzed ribosylation and obtained results qualitatively similar to those reported for DE and DE fragment A, i.e., inhibitory potency of the order adenine >> adenosine > AMP $\cong$ ADP $\cong$ ATP (data not shown). A4P was no more potent at inhibiting ribosylation catalyzed by either toxin than was ATP. The effects of pyrophosphate and tripolyphosphate on ribosylation have not been previously described and are shown in Figure 7. Since it is known that ionic strength can inhibit DE fragment A-catalyzed ribosylation (6), the data were plotted in terms of ionic strength rather than molarity. Although both phosphate compounds inhibit toxin-catalyzed ADP-ribosylation, the level of inhibition appears similar to that induced by NaCl. Thus, phosphate oligomer inhibition of in vitro ribosyl transferase activity is probably a nonspecific ionic strength effect.

DISCUSSION

Although nucleotides protect mammalian cells from both PE and DE, our data indicate that the mechanisms involved are different for each toxin. Results suggest that nucleotide protection of cells from PE is mediated solely by the oligomeric phosphate moieties.

Most convincing are the data showing that all the nucleotide triphosphates have PE-protective potencies identical to that of tripolyphosphate (Fig. 3B). This, coupled with the fact that none of the tested compounds exhibited PE protection at concentrations < 1 mM, implies a more nonspecific effect than that responsible for DE protection.

The observation that nucleotides and phosphate oligomers will protect L-929, but not HeLa, cells from PE may indicate that there is more than one mechanism by which PE enters the cell and is "activated." Support for this proposal exists in the demonstration that the in vitro ribosylation activity of PE may be markedly increased in two different manners, by treatment with denaturant and reductant (14) or by fragmentation (31). Possibly phosphates are able to block only one of these "activation mechanisms" (if either occur in vivo) and thus protect only the cell line for which that particular mechanism of intoxication is operable. In any case, it is clear that the choice of cell line may be critical in studies of this type, since L-929 is the only one of seven PE-responsive lines examined which was protected by nucleotides while all DE-responsive lines were. Indeed, in our recently published chemical and drug protection study (16), we used only HeLa and HEp-2 cells, both relatively insensitive to PE. We have since reinvestigated the drugs tested in that work using highly PE-sensitive L-929 cells and obtained comparable results in nearly all cases. The single exception, NH_4Cl, was able to protect L-929, but not HeLa or HEp-2, cells from the cytotoxic action of PE.

Although at high concentrations, phosphates alone can provide protection from DE as well as from PE (Figs. 2A and B), the characteristics of nucleotide protection from DE are significantly different. There is a specificity of the nitrogenous base for protective potency. CTP, for example, was not significantly different from tripolyphosphate in protective activity, while TTP was at least 20-fold more active. Surprisingly, in view of the clear differences in potencies of the common purine and pyrimidine nucleotide triphosphates, chemical substitutions on the base did not detectably alter the DE-protective potency relative to the parent compounds (Table 2). Moreover, potency was not affected by replacement of the ribose with other pentoses, indicating that, unlike the base, the type of sugar moiety does not greatly influence protective level. The length of the nucleotide phosphate chain is very important as was demonstrated in Fig. 1A. There, a several log increase in potency was observed as the chain length was increased from one to four phosphates. This pattern was also observed with guanine, thymine, inosine, and uridine nucleotides (data not shown). Protection was never obtained with any nucleoside or free base. Thus it appears that nucleotide protection from DE has two components: one dependent on an intact nucleotide, the other on phosphates only.

The similarity of the dose-response curves for phosphate-mediated protection from PE and DE may indicate a common locus of action for the phosphate oligomers. At present, the only known common feature of PE and DE intoxication is their similar ADP-ribosyl transferase activity. A direct in vitro test of pyrophosphate and tripolyphosphate

effects on toxin-catalyzed ribosylation demonstrated rather similar, probably nonspecific inhibition (Fig. 7). However, assuming that inhibition of ribosylation is roughly proportional to protection of cells, it would require intracellular pyro- or tripolyphosphate concentrations of 5 mM or greater to account for the observed protective effect. It seems extremely unlikely that cultured cells could achieve such high intracellular concentrations. Furthermore, neither phosphate compound protects HeLa cells from PE, although both effectively prevent DE intoxication. Since previous studies have indicated that EF-2 from all cell sources is ribosylated similarly by both toxins (5, 18), it follows that a block of cytotoxicity at the level of ribosylation should affect both toxins in the same manner. Since such is not the case in HeLa cells, it is highly unlikely that the phosphate compounds protect cells by inhibiting in vivo ribosylation.

Several laboratories have reported that exogenous nucleotides interact with, or affect binding to, cell membranes. Rodbell and associates (26-28) showed that exogenous nucleotides can inhibit the binding of glucagon to cell membranes; Lefkowitz et al. (13) demonstrated inhibition of the binding of β-adrenergic agonists to their membrane receptor by exogenous purine nucleotides. In contrast, Ohamura and Terayama (20) found that exogenous nucleotides enhanced the binding of prostaglandin E_1 to membranes. Nucleotide-membrane interactions have also been shown to occur via nucleoside triphosphatase "ectoenzymes" located at the outer surfaces of cultured mammalian cells (30).

Similarly, exogenous nucleotides can affect cellular physiological processes. Cohn and Parks (4) showed that nucleotides can stimulate vesicle formation and pinocytosis in mouse macrophages; North (19)

found that exogenous ATP accelerated phagocytosis and cellular spreading of guinea pig macrophages. More recently, it has been shown that exogenous ATP may bring about a conformational change in the Ca^{++}-dependent ATPase of the sarcoplasmic reticulum (3). We find this work particularly interesting since we have evidence implicating the Ca^{++}-, Mg^{++}-dependent ATPase in DE intoxication (16).

The molecular events responsible for nucleotide protection from DE are unclear. The protection is somewhat toxin-specific in that nucleotides did not protect HeLa cells from PE or the plant toxin abrin (data not shown). It seems unlikely that a nucleotide-mediated inhibition of intracellular ribosylation is responsible for the protection from DE. The pattern of cellular protective potency (A4P > ATP > ADP > AMP) is diametrically opposed to the potency pattern for inhibition of in vitro ribosylation (10). In any case, since it is generally believed that nucleotides do not cross the cell membrane, the (intracellular) site of ribosylation should be inaccessible to these compounds. Thus, the locus of nucleotide action is probably the cell membrane at the attachment or internalization stages of intoxication.

ACKNOWLEDGMENTS

The authors would like to express their appreciation to John Kondig for supplying the cells used in this work and to Dennis Leatherman and Bruce Loverock for their excellent technical assistance. We would also like to acknowledge many helpful comments from Stephen Leppla.

LITERATURE CITED

1. Birnbaumer, L., S. L. Pohl, and M. Rodbell. 1971. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. II. Comparison between glucagon- and fluoride-stimulated activities. J. Biol. Chem. 246:1857-1860.

2. Bjorn, M. J., M. L. Vasil, J. C. Sadoff, and B. H. Iglewski. 1977. Incidence of exotoxin production by Pseudomonas species. Infect. Immun. 16:362-366.

3. Coan, C. R., and C. Inesi. 1977. Ca^{2+}-dependent effect of ATP on spin-labeled sarcoplasmic reticulum. J. Biol. Chem. 252:3044-3049.

4. Cohn, Z. A., and E. Parks. 1967. The regulation of pinocytosis in mouse macrophages. III. The induction of vesicle formation by nucleosides and nucleotides. J. Exp. Med. 125:457-466.

5. Collier, R. J. 1975. Diphtheria toxin: mode of action and structure. Pacteriol. Rev. 39:54-85.

6. Collier, R. J., and J. Kandel. 1971. Structure and activity of diptheria toxin. I. Thiol-dependent dissociation of a fraction of toxin into enzymically active and inactive fragments. J. Biol. Chem. 246:1496-1503.

7. Colowick, S. P., and F. C. Womack. 1969. Binding of diffusible molecules by macromolecules: rapid measurement by rate of dialysis. J. Biol. Chem. 244:774-777.

8. Delange, R. J., R. E. Drezin, and R. J. Collier. 1976. Amino-acid sequence of Fragment A, an enzymically active fragment from diphtheria toxin. Proc. Nat. Acad. Sci. USA 73:69-72.

9. Gill, D. M., A. M. Pappenheimer, Jr., and T. Uchida. 1973. Diphtheria toxin, protein synthesis, and the cell. Fed. Proc. 32:1508-1515.

10. Goor, R. S., and E. S. Maxwell. 1970. The diphtheria toxin-dependent adenosine diphosphate ribosylation of rat liver aminoacyl transferase. II. General characteristics and mechanism of the reaction. J. Biol. Chem. 245:616-623.

11. Iglewski, B. H., and D. Kabat. 1975. NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Nat. Acad. Sci. USA 72:2284-2288.

12. Kandel, J., R. J. Collier, and D. W. Chung. 1974. Interaction of Fragment A from diphtheria toxin with nicotinamide adenine dinucleotide. J. Biol. Chem. 249:2088-2097.

13. Lefkowitz, R. J., D. Mullikin, and M. G. Caron. 1976. Regulation of β-adrenergic receptors by guanyl-5'-yl imidodiphosphate and other purine nucleotides. J. Biol. Chem. 251:4686-4692.

14. Leppla, S. H. 1976. Large-scale purification and characterization of the exotoxin of Pseudomonas aeruginosa. Infect. Immun. 14: 1077-1086.

15. Liu, P. V. 1976. Biology of Pseudomonas aeruginosa. Hosp. Pract. 11:139-147.

16. Middlebrook, J. L., and Dorland, R. B. 1977. Differential chemical protection of mammaliam cells from the exotoxins of Corynebacterium diphtheriae and Pseudomonas aeruginosa. Infect. Immun. 16:232-239.

17. Middlebrook, J. L. and R. B. Dorland. 1977. Serum effects on the response of mammalian cells to the exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheriae. Can. J. Microbiol. 23:175-182.

18. Middlebrook, J. L. and R. B. Dorland. 1977. Response of cultured mammalian cells to the exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheriae: differential cyxotoxicity. Can. J. Microbiol. 23:183-189.

19. North, R. J. 1968. The uptake of particulate antigens. J. Reticuloendothel. Soc. 5:203-229.

20. Okamura, N., and H. Terayama. 1977. Prostaglandin receptor-adenylate cyclase system in plasma membranes of rat liver and ascites hepatomas, and the effect of GTP upon it. Biochim. Biophys. Acta 465:54-67.

21. Pappenheimer, A. M., Jr., and D. M. Gill. 1973. Diphtheria. Science 182:353-358.

22. Pavlovskis, O. R., and F. B. Gordon. 1972. Pseudomonas aeruginosa exotoxin: effect on cell cultures. J. Infect. Dis. 125:631-636.

23. Pavlovskis, O. R., and A. M. Shackelford. 1974. Pseudomonas aeruginosa exotoxin in mice: localization and effect on protein synthesis. Infect. Immun. 9:540-546.

24. Pollack, M., N. S. Taylor, and L. T. Callahan III. 1977. Exotoxin production by clinical isolates of Pseudomonas aeruginosa. Infect. Immun. 15:776-780.

25. Randerath, K. and Randerath, E. 1967. Thin-layer separation methods for nucleic acid derivitives, p. 323-347 In L. Grossman and K. Moldave (ed.), Methods In Enzymology XII A. Academic Press, N. Y.

26. Rodbell, M., L. Birnbaumer, S. L. Pohl, and H. M. J. Krans. 1971. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanyl nucleotides in glucagon action. J. Biol. Chem. 246: 1877-1882.

27. Rodbell, M., H. M. J. Krans, S. L. Pohl, and L. Birnbaumer. 1971. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. III. Binding of glucagon: method of assay and specificity. J. Biol. Chem. 246:1861-1971.

28. Rodbell, M., H. M. J. Krans, S. L. Pohl, and L. Birnbaumer. 1971. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. IV. Effects of guanyl nucleotides on binding of ^{125}I-glucagon. J. Biol. Chem. 246:1872-1876.

29. Sober, H. A. (ed.). 1970. Handbook of biochemistry and selected data for molecular biology, 2nd ed. p. C8-C98. The Chemical Rubber Co., Cleveland, Ohio.

30. Trams, E. G., and C. J. Lauter. 1974. On the sidedness of plasma membrane enzymes. Biochim. Biophys. Acta 345:180-197.

31. Vasil, M. L., D. Kabat, and B. H. Iglewski. 1977. Structure-activity relationships of an exotoxin of Pseudomonas aeruginosa. Infect. Immun. 16:353-361.

TABLE 1. List of chemicals, their abbreviations, and sources

Chemical	Abbreviation	Source[a]
Acetyl coenzyme A	Acetyl CoA	PL
Adenosine-5'-tetraphosphate	A4P	S
Adenosine-5'-triphosphate	ATP	S,C,PL
Adenosine-5'-diphosphate	ADP	S
Adenosine-5'-monophosphate	AMP	S
Adenosine	---	S
Adenine	---	S
Adenosine 3':5'-cyclic-monophosphoric acid	cAMP	S
N^6,O^2-dibutyryl adenosine 3':5'-cyclic-monophosphoric acid	Dibutyryl cAMP	S
Adenosine diphosphoribose	ADPR	PL
Adenine 9-β-D-arabinofuranoside-5'-triphosphate	Ara-ATP	PL
Adenylyl-(3'-5')-adenosine	ApA	PL
Adenylyl-(3'-5')-cytidine	ApC	PL
α-β-Methyleneadenosine-5'-triphosphate	AMP-MPP	PL
β-γ-Methyleneadenosine-5'-triphosphate	AMP-PMP	PL
5'-Adenylylimido-diphosphate	AMP-PNP	PL
Cytidine-5'-triphosphate	CTP	S,C,PL
Cytidine-5'-diphosphate	CDP	S
Cytidine-5'-monophosphate	CMP	S
Cytidine	---	S
Cytosine	---	S
Cytidylyl-(3'-5')-adenosine	CpA	PL
5-Methylcytosine	---	S
Cytosine-β-D-arabinofuranoside-5'-triphosphate	Ara-CTP	PL
2'-Deoxycytidine-5'-triphosphate	dCTP	S
5-Bromo-2'-deoxycytidine-5'-triphosphate	5Brd-CTP	PL
Guanosine-5'-tetraphosphate	G4P	S
Guanosine-5'-triphosphate	GTP	S,C,PL
Guanosine-5'-diphosphate	GDP	S
Guanosine-5'-monophosphate	GMP	S

Guanosine	---	S
Guanine	---	S
Guanosine 3':5'-cyclic-monophosphoric acid	cGMP	S
$N^2,O^{2'}$-dibutyryl guanosine 3':5'-cyclic-monophosphoric acid	dibutyryl cGMP	S
2'-Deoxyguanosine-5'-triphosphate	dGTP	S
Inosine-5'-triphosphate	ITP	S,C,PL
Inosine-5'-diphosphate	IDP	S
Inosine-5'-monophosphate	IMP	S
Inosine	---	S
Inositol	I	S
Inositol monophosphate	IP	S
Inositol hexaphosphate	IHP	PL
Inositol hexasulfate	IHS	C
Hypoxanthine	---	S
(1,N^6-Etheno) Nicotinamide adenine dinucleotide	ε-NAD	PL
Phosphorylribosyl-pyrophosphate	PRPP	S
Thymidine-5'-triphosphate	TTP	S,C
Thymidine-5'-diphosphate	TDP	S
Thymidine-5'-monophosphate	TMP	S
Thymidine	---	S
Thymine	---	S
Trifluorodeoxythymidine-5'-triphosphate	F_3d-TTP	PL
Uridine-5'-triphosphate	UTP	S,C,PL
Uridine-5'-diphosphate	UDP	S
Uridine 5'-monophosphate	UMP	S
Uridine	---	S
Uracil	---	S
2'-Deoxyuridine-5'-triphosphate	dUTP	S
5-Bromo-2'-deoxyuridine-5'-triphosphate	5 Br-dUTP	PL
Xanthosine-5'-triphosphate	XTP	S

[a]Sources are as follows: S, Sigma Chemical Co., St. Louis, Mo.; C, Calbiochem, La Jolla, Cal.; PL, PL Biochemicals, Inc., Milwaukee, Wis.

Table 2. DE-protective potency of nucleoside phosphate analogs

Group	Compound	Effect
A	Ara-ATP	+++
	dATP	+++
	Ara-CTP	+
	dCTP	+
	dGTP	+++
	dITP	+++
	dUTP	+++
B	8Br-ATP	+++
	5Br-dCTP	+
	F_3dTTP	+++
	5Br-dUTP	+++
	XTP	++
C	ApA	+
	ApC	+
	CpA	+
	Poly A	+
	Poly C	+
	cAMP	0
	Dibutyryl cAMP	0
	cGMP	0
	Dibutyryl cGMP	0
D	ADPR	+
	PRPP	+
	Acetyl CoA	+
	CoA	0
	NAD	0
	cNAD	0
	Heparin	0
	IHS	+++
	IHP	+++
	IP	+
	I	0

FIGURE LEGENDS

Fig. 1. Protection from DE and PE by adenine nucleotides. Effects of nucleotides on DE and PE cytotoxicity were determined as described in Materials and Methods. A: HeLa cells challenged with 10 ng/ml DE: A4P (◇), ATP (○), ADP (□), AMP (△), buffer (*). B: L929 cells challenged with 10 ng/ml PE: A4P (◇), ATP (○), ADP (□), AMP (△), buffer (*).

Fig. 2. Protection from DE and PE by oligomeric phosphates. Effects of oligomeric phosphates on DE and PE cytotoxicity were determined as described in Materials and Methods. A: HeLa cells challenged with 10 ng/ml DE: ATP (○), pyrophosphate (□), tripolyphosphate (△). B: L929 cells challenged with 10 ng/ml PE: ATP (○), pyrophosphate (□), tripolyphosphate (△).

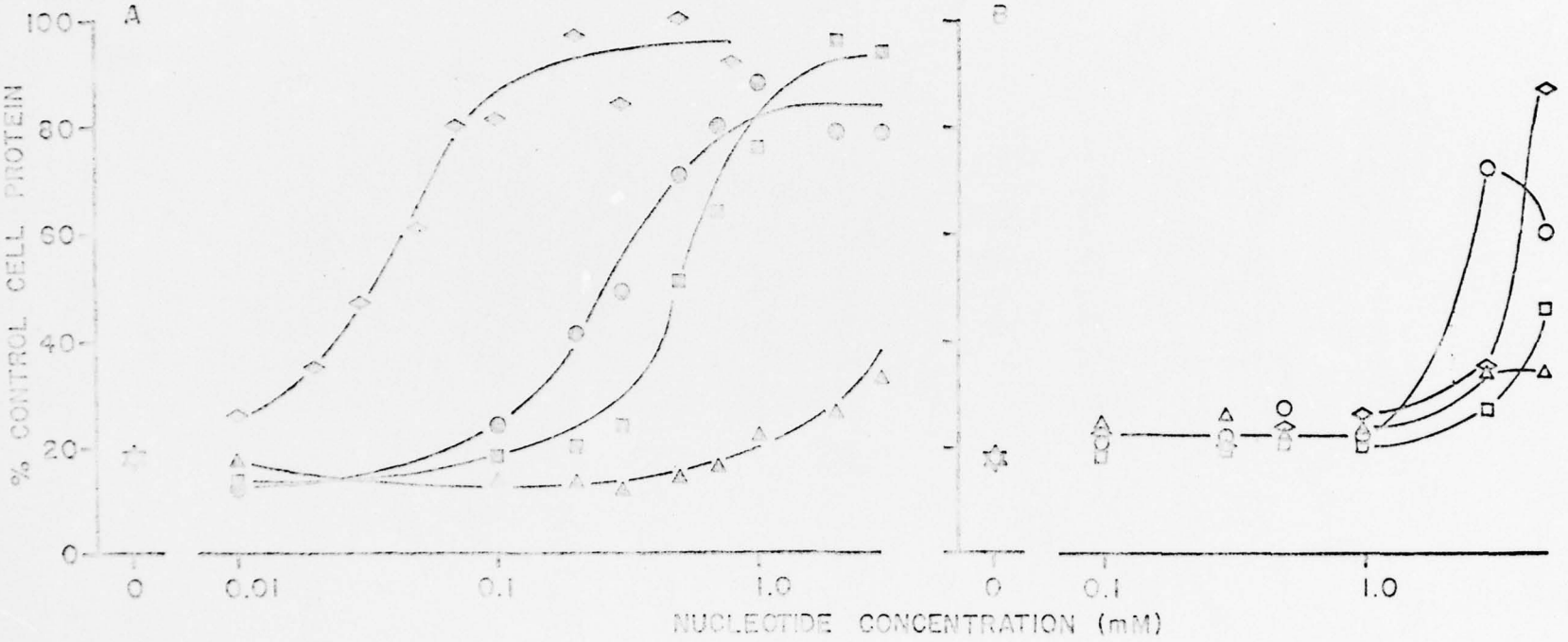
Fig. 3. Protection from DE and PE by triphosphate nucleotides. Effects of nucleotide triphosphates on DE- and PE-induced cytotoxicity were determined as described in Materials and Methods. A: HeLa cells challenged with 10 ng/ml DE: ATP (○), CTP (□), ITP (◇), GTP (○), TTP (□), UTP (△). B: L-929 cells challenged with 10 ng/ml PE: same symbol notation as part A and tripolyphosphate, (△).

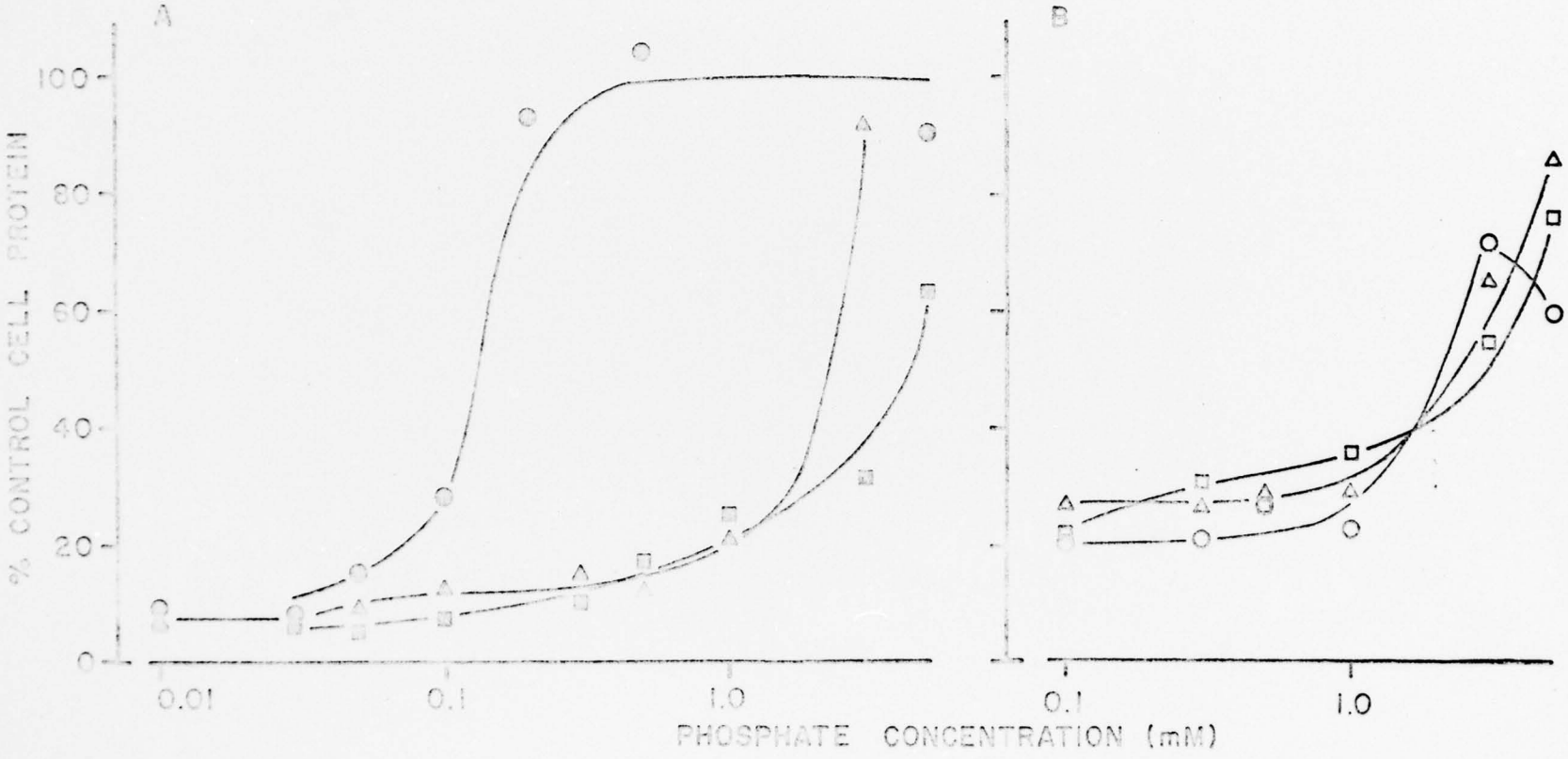
Fig. 4. Protection from DE by nucleotide components. The effects of 1 mM concentrations of the agents indicated were determined by the standard cytotoxicity test using HeLa cells challenged with 10 ng/ml DE. Error bars show standard error of mean.

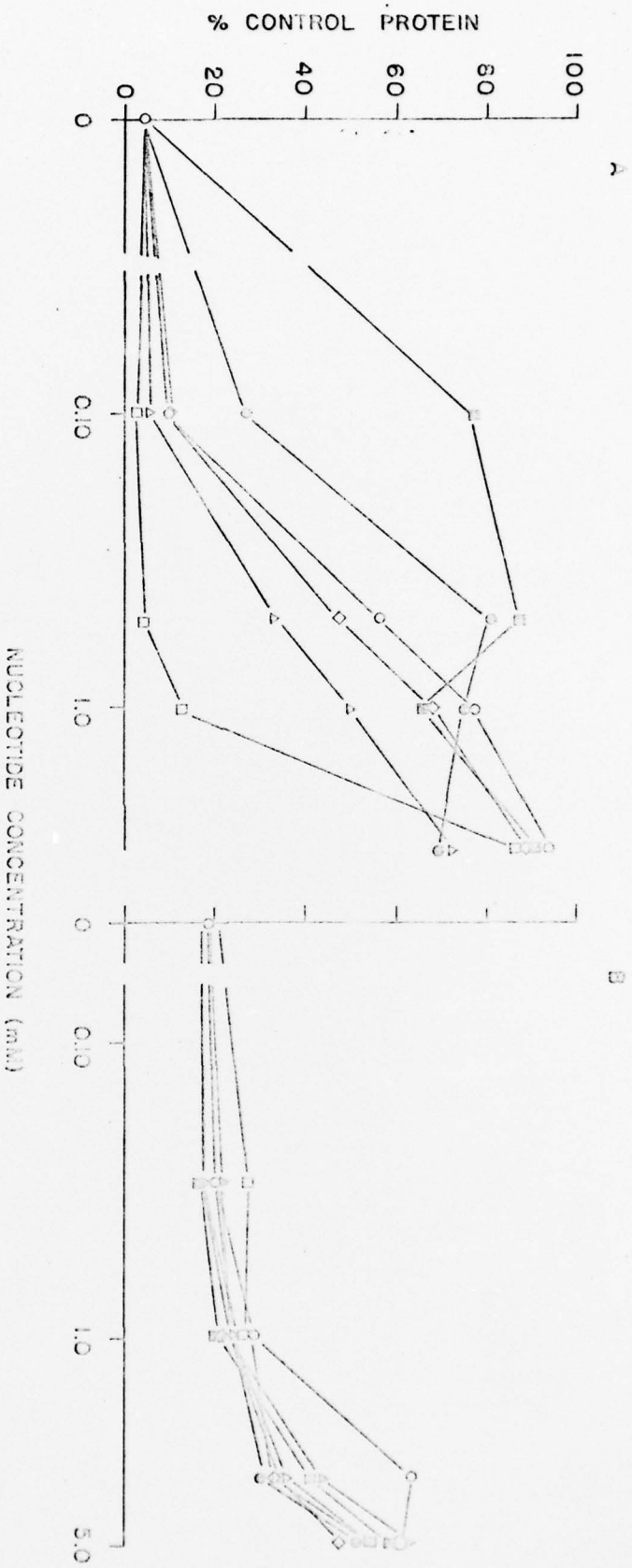
Fig. 5. Kinetics of nucleotide protection. (○, -2-1/2 to -1/2 h) HeLa cells preincubated with 1 mM ATP for times indicated prior to initiation of 3-h 10 ng/ml DE challenge. (○, 0 h), simultaneous addition of 1 mM ATP and 10 ng/ml DE for 3-h challenge. (●, +1/2 to +3 h), 1 mM ATP added at indicated time after initiation of 3-h challenge with 10 ng/ml DE. (Band), 3-h challenge of 10 ng/ml DE. (△) 100 ng/ml DE or (▲) 100 ng/ml DE + 10 mM ATP preincubated at 37°C for times indicated prior to addition of 1/10 volumes to cells for 3 h challenge. In each case toxin challenge terminated by washing cells and cytotoxicity determined as usual.

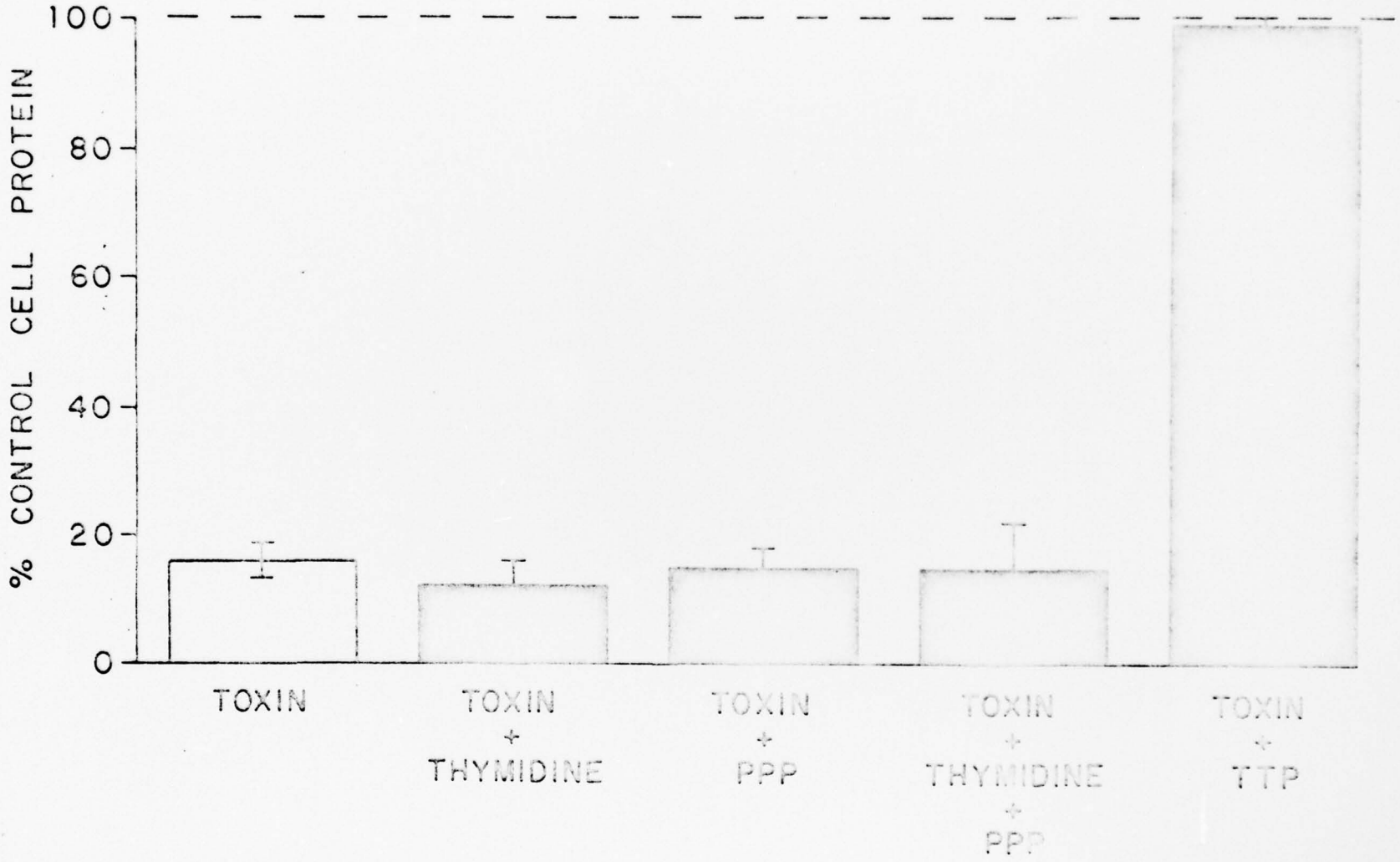
Fig. 6. Protection from DE by nonhydrolyzable ATP analogs. Effects of 1 mM ATP and 1 mM analogs on the HEp-2 cell cytotoxicity of 10 ng/ml DE were determined as described in Materials and Methods. Error bars show standard errors of the mean.

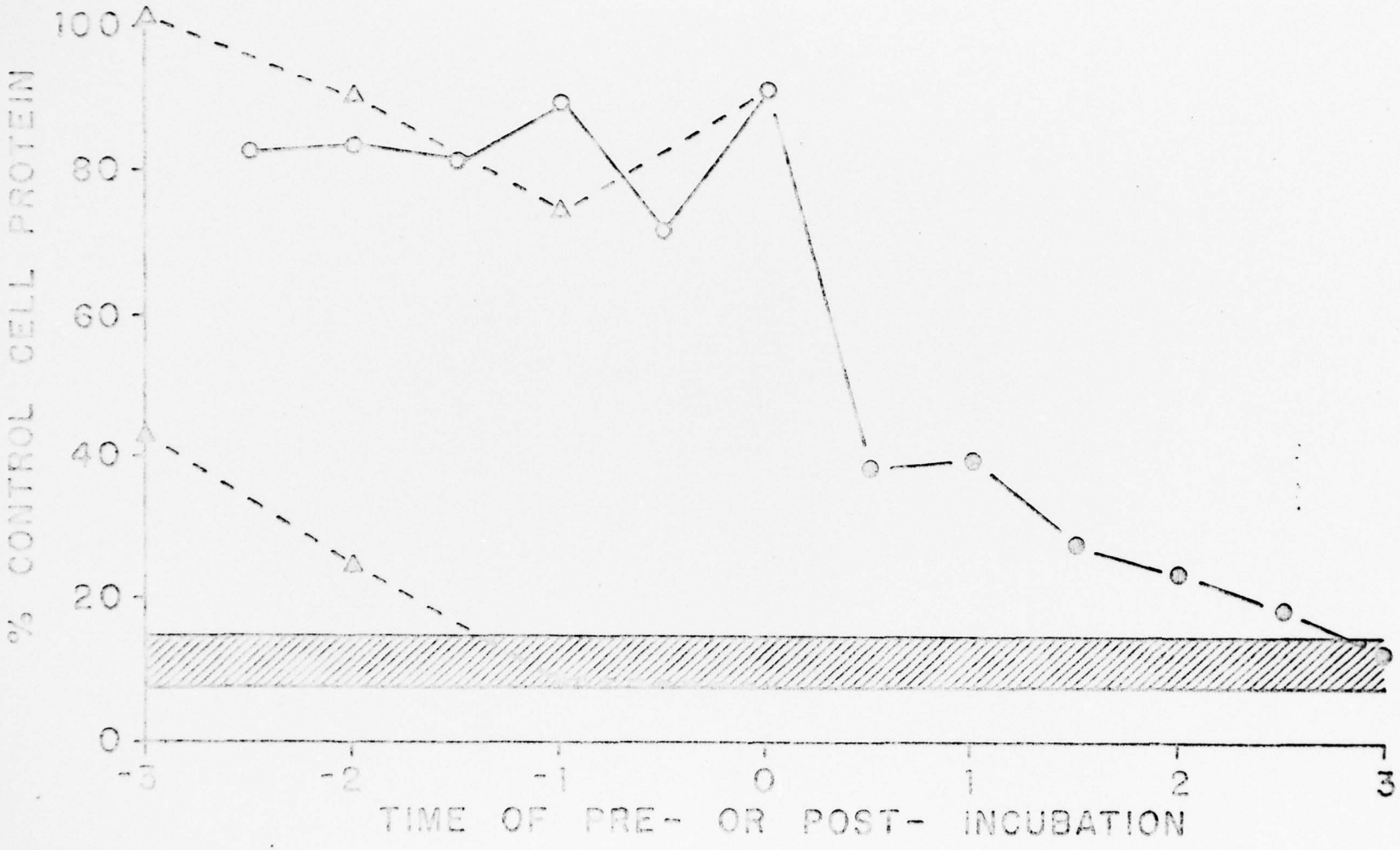
Fig. 7. Effects of oligomeric phosphates on ADPR-transferase activities. The effects of pyro- and tripolyphosphate on DE fragment A- and "activated PE"-catalyzed ribosylation were determined as described in Materials and Methods. Results are plotted as % of the control with no phosphate A: DE-fragment A, 0.3 µg/ml, control 2070 cpm. (○) pyrophosphate; (□) tripolyphosphate; (△), NaCl. B: "Activated PE," 10 µg/ml, control 8070 cpm. (○), pyrophosphate; (□), tripolyphosphate; (△), NaCl.

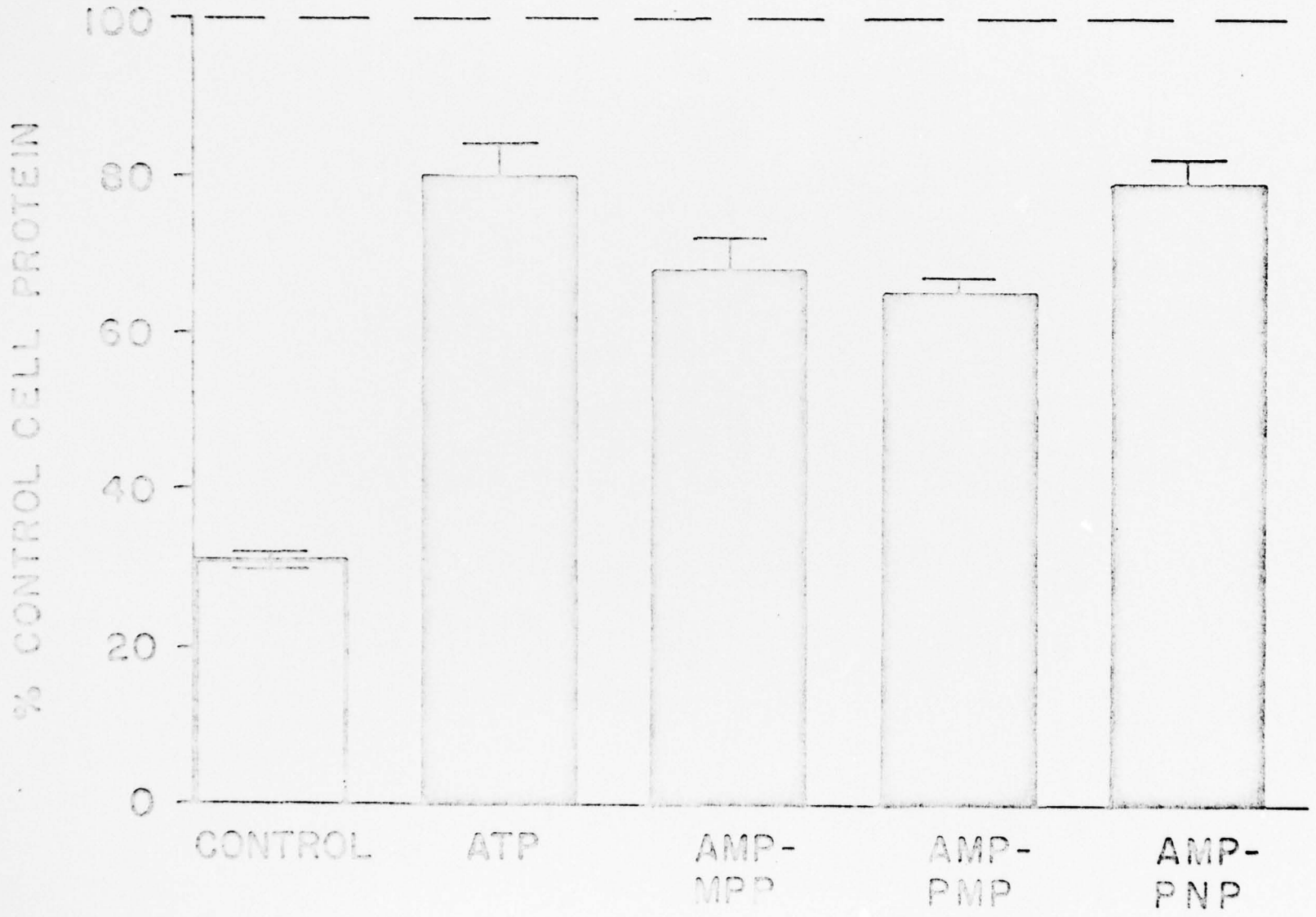

A
B
100
80
60
40
20
0
% CONTROL CELL PROTEIN
0
0.01
0.1
1.0
0
0.1
1.0
NUCLEOTIDE CONCENTRATION (mM)

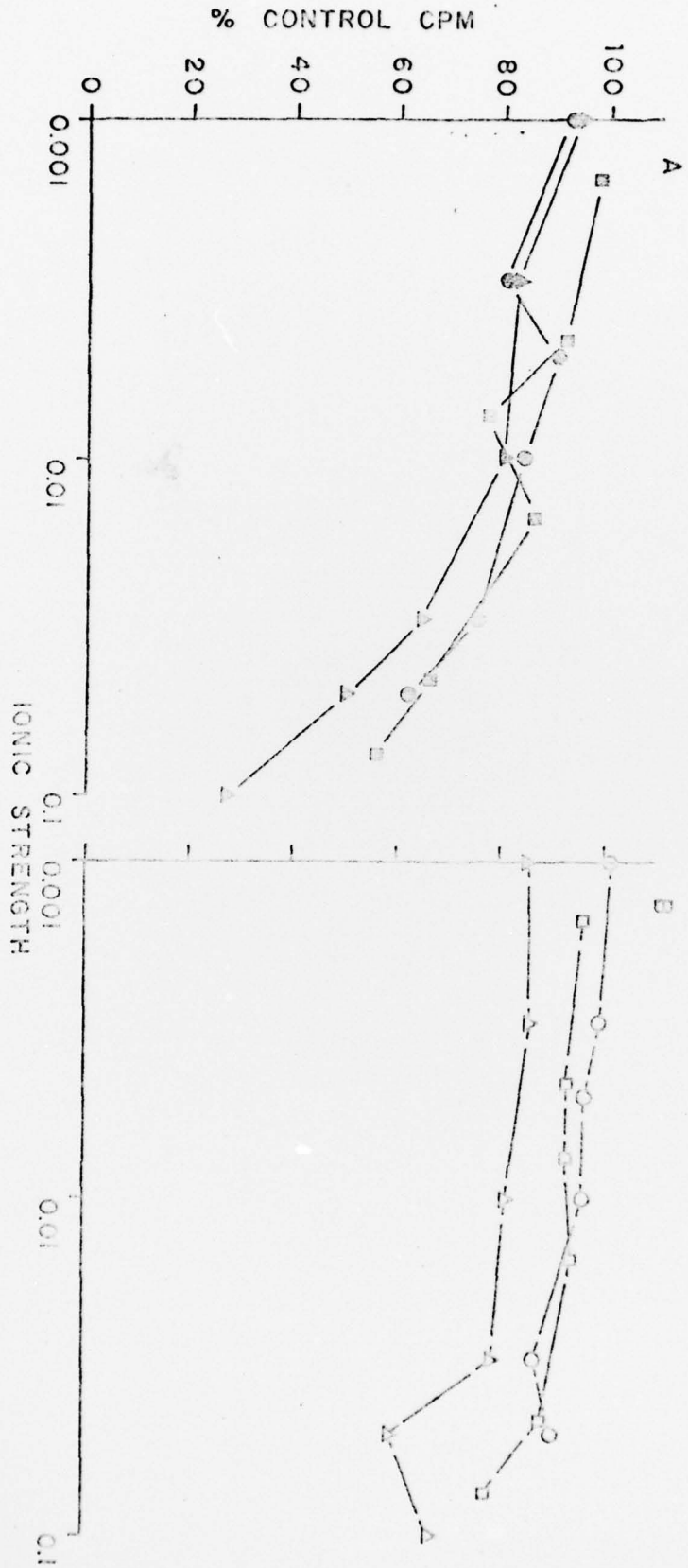

A
B
100
80
60
40
20
0
% CONTROL CELL PROTEIN
0.01
0.1
1.0
0.1
1.0
PHOSPHATE CONCENTRATION (mM)


% CONTROL PROTEIN
0
20
40
60
80
100
A
0
0.10
1.0
B
0
0.10
1.0
5.0
NUCLEOTIDE CONCENTRATION (mM)


% CONTROL CELL PROTEIN
100
80
60
40
20
0
TOXIN
TOXIN + THYMIDINE
TOXIN + PPP
TOXIN + THYMIDINE + PPP
TOXIN + TTP


% CONTROL CELL PROTEIN
100
80
60
40
20
0
-3
-2
-1
0
1
2
3
TIME OF PRE- OR POST- INCUBATION


% CONTROL CELL PROTEIN
100
80
60
40
20
0
CONTROL
ATP
AMP-MPP
AMP-PMP
AMP-PNP


% CONTROL CPM
0
20
40
60
80
100
A
B
0.001
0.01
0.1
IONIC STRENGTH